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Combination of microstereolithography and electrospinning to produce membranes equipped with niches for corneal regeneration

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SHORT ABSTRACT: Here we report a combination of microstereolithography and electrospinning for the production of PLGA (Poly(lactide-co-glycolide)) corneal biomaterial devices equipped with microfeatures. The inclusion of these microfeatures within PLGA membranes gives an improvement in epithelial cell performance and consequently corneal regeneration.

LONG ABSTRACT: Corneal problems affect millions of people worldwide reducing their quality of life significantly. Corneal disease can be caused by illnesses such as Aniridia or Steven Johnson Syndrome as well as by external factors such as chemical burns or radiation. Current treatments are (i) the use of corneal grafts and (ii) the use of stem cell expanded in the laboratory and delivered on carriers (e.g.amniotic membrane); these treatments are relatively successful but unfortunately they can fail after 3-5 years. There is a need to design and manufacture new corneal biomaterial devices able to mimic in detail the physiological environment where stem cells reside in the cornea. Limbal stem cells are located in the limbus (circular area between cornea and sclera) in specific niches known as the Palisades of Vogt. In this work we have developed a new platform technology which combines two cutting-edge manufacturing techniques (microstereolithography and electrospinning) for the fabrication of corneal membranes that mimic to a certain extent the limbus. Our membranes contain artificial micropockets which aim to provide cells with protection as the Palisades of Vogt do in the eye.

INTRODUCTION

The cornea, the avascular central outer most tissue of the eye, is one of the most important tissues involved in vision[1]. There are several types of cells that maintain the function of the cornea. The top outermost layer of the cornea comprises of epithelial cells which can be about 5-7 layers in thickness[2]. This layer prevents bacterial invasion into the cornea[3] and allows entry of oxygen[4]. It has been reported that the stem cells of the corneal epithelium lie in niches or crypts at the peripheral

region of the cornea known as the limbus[5, 6]. As the stem cells divide, the daughter cells also known as transient amplifying cells travel out of the niches and as division continues the cells move centripetally inwards and upwards resulting in terminally differentiated cells at the central corneal region[7, 8]. These cells are routinely wiped away with the blink of the eye exposing newer cells underneath[9].

In addition to being the location of the epithelial stem cells, the limbus also plays a role in keeping the vascularised conjunctiva away from the cornea region[10]. Damage to the limbus could be caused by thermal/chemical burns, radiation and also genetic diseases[10]. When this happens, the limbus barrier is broken down allowing the conjunctival cells to move onto the cornea, vascularising the region, causing pain and blindness in some cases. The condition is known as limbal stem cell deficiency (LSCD) [10].

At present the most common method of treatment is to use human amniotic membrane from a tissue bank and culture limbal epithelial cells on its surface[11, 12]. Once a monolayer has formed, the amniotic membrane is glued cell-side up onto the damaged cornea which has all the conjunctival cells and scar tissue surgically removed from it prior to this cell transplantation[12]. The amniotic membrane degrades within weeks to months leaving the epithelial cells attached to the denuded area to regenerate the epithelium[13, 14]. This technique has been successful in restoring vision however there are still a few practical issues which restrict its widespread uptake clinically. As the amniotic membrane is human tissue it needs to undergo screening using good tissue banking procedures before being used for cell transplantation on patients. This screening only lowers the risk of transmission of diseases but cannot completely eliminate it[15]. In addition to this there have been reports of variability in the performance of the amniotic membrane due to inter donor variation[16, 17] and different processing methods[17, 18]. Alongside the small risk of disease transmission there is the requirement for surgical centres to have access to well-run tissue banks, not available to all.

Although the amniotic membrane is relatively successful, there is a need for the development of new synthetic biodegradable cell carrier alternatives for the treatment of corneal disease. Synthetic carriers would overcome the need for banking procedures as well as eliminating the small risk of disease transmission and inter-donor variability. In this sense, materials such as polyethylene glycol [19, 20] and PLGA [21, 22] have been studied.

In developing a synthetic alternative to the human amniotic membrane there is also the possibility to design into it desirable features to hopefully help the survival of the cultured cells. The inclusion of microfeatures within biomaterial devices for the specific control of cell behavior is an emerging area of interest. Many authors have reported work towards the development of artificial stem cells niches[23-28]. Our group has recently reported the creation of a microfabricated PEGDA fibronectin-biofunctionalised artificial limbus for the delivery of limbal epithelial cells[20] and a methodology for the fabrication of electrospun biodegradable membranes containing microfabricated pockets for the support of limbal epithelial cells [29].
The aim of this work is to develop a new manufacturing technology for the development of biomaterial devices containing microfeatures which mimic to an

extent the microenvironments in which stem cells reside in the body. We have developed a technique which combines microstereolithography and electrospinning that allows the fabrication of biodegradable microstructured membranes that show great potential for tissue regeneration applications.
It is important to notice that although in this work we have applied this technique to the fabrication of rings for corneal regeneration, the technology can be applied to the fabrication of devices for the regeneration of a broad range of epithelial tissues e.g. skin, oral mucosa, intestine, respiratory and bladder epithelia. Specifically, in this study we have developed a synthetic biodegradable membrane which functions in a similar manner to the amniotic membrane to deliver cells to the cornea. This membrane contains micropockets designed to provide some physical protection for limbal epithelial cells from the external environment while the daughter cells regenerate the cornea. Finally, we have established a packaging protocol which allows these membranes to be stored at -20 °C for more than 6 months without showing any signs of breakdown.

PROTOCOL

Eyes from New Zealand white rabbits were obtained from Hook farm, Hook, Hampshire, UK. The rabbits were sacrificed for human consumption and the eyes which would have been waste were used in the following experiments.

1. Fabrication of PLGA biodegradable membranes equipped with micropockets

The ring scaffolds were created by a combination of microstereolithography and electrospinning techniques recently reported by our group[29]. In essence, the process can be summarized in two parts (i) creation of PEGDA templates by microsterelithography and (ii) electrospinning onto the templates for reproduction of the underlying PEGDA structure (in this case a microfabricated ring). This two steps are described in detail below (Figures 1 and 2).

1.1. Fabrication of PEGDA templates by microstereolithography (microSL)

The rings were fabricated using a 2-layer model. The first layer (L1) being the base of the structure and the second layer (L2) presenting 6 micropockets with horseshoe morphologies in a range of sizes from 300-500 µm. The fabrication of PEGDA rings was recently described by our group[20].
The laser beam was expanded using a telescopic lens arrangement and then projected onto a computer programmable digital multimirror device (UV-enabled DMD starter kit, Texas Instruments). The DMD reflects the image (in our case a ring) onto a mirror (Thorlabs) via a 10 cm focal length tube lens (Thorlabs). The image is then directed by the silver-coated mirror into a vial containing the photocurable polymer (PEGDA).
1. In a dark glass vial, mix polyethylene glycol diacrylate (PEGDA, Mn= 250 g/mol, Aldrich) with 1% w/w Camphorquinone (Aldrich), a photoinitiator, on a magnetic stirrer for 20 min.
2. Design the 2 layers of the ring template (L1 and L2) using Corel Draw, paint or any other suitable program. L1=black circle; L2=black circle with white microfeatures.
3. Upload the images into the ALP3-basic software previously installed on the PC.
4. Adjust and carefully clean the optics of the microstereolithography set up.
5. Put 300 µl of the PEGDA mixture into a well (use a 12-well tissue culture plate). Make sure the wells are pre-coated with Teflon or other non-stick material for easy removal of the structure after curing.
6. Switch on the blue laser (MBL-III 473 nm; 150 mW) and upload L1. Irradiate the first layer for 60 s.
7. Add to the well 250 µl more of the PEGDA and irradiate L2 for 60 s.
8. Remove the uncured polymer and wash the structure with isopropanol overnight.

1.2. Fabrication of Biodegradable PLGA membranes using electrospinning

The PEGDA rings were used as templates for the spinning of PLGA and the creation of PLGA rings by the PLGA reproducing the underlying topography as it is spun over these templates.

1. Distribute the PEGDA rings on an electroplated aluminium sheet (12 cm x20 cm) for creating a static electrospinning collector. Attach the rings using conductive carbon tape.

2. Prepare the polymer solution for spinning. Dissolve PLGA (50/50 DL-lactide (52 mol%): glycolide (48 mol%), 44 kg/mol, Purac) in dichloromethane (DCM) at 20% w/w concentration (stir overnight before use).
3. Place four insulin syringes (blunt ended, 0.8 cm inner diameter needles (Intertronics, UK) on a syringe pump. Load 2.5 ml of PLGA solution in each syringe.
4. Electrospin using a 30 µl/min flow rate and voltages ranging from 12 to 15 kV. Leave a distance between the needles and the collector of 15 cm.
5. Electrospin for 1 hour and 30 min and finally carefully peel the PLGA electrospun sheet from the collector.

2. Long-term Storage of PLGA microfabricated membranes

As a requirement for undertaking future clinical studies one of our objectives was to achieve long term storage of the sterilised PLGA membranes with microfabricated rings. The rings should be stored in conditions to avoid undesirable uptake of humidity and consequent fiber degradation. We explored the contribution of vacuum sealing and the use of different storage bags (table 1). Accordingly this section describes the packaging, irradiation, storage and stability of the membranes.
For this purpose, the PLGA ring membranes were fabricated by The Electrospinning Company Ltd. (Oxford). The rings were placed in 12 well plates and they were sent for sterilization to Applied Sterilisation Technologies (Synergy Health Laboratory Services (SHLS), Abergavenny UK), an accredited external company which has ISO-9001, ISO-11137 and ISO-13485 accreditation and ɣ-irradiated at an external dose range of 25-40 KGy.

1. Mount the membrane in a small container (Plastic petri dish) and place it inside a medical grade bag (PET/Foil/LDPE; Riverside Medical Ltd. Derby, UK).
2. Use filter paper to create small filter bags for desiccants. Fill 3 different filter paper bags with 1gr of silica orange, cobalt (II) chloride and copper (II) sulphate respectively. Put the three bags of desiccant inside the medical grade bag along with the electrospun membrane.
3. Add to the bag a commercially available six spot humidity indicator card (SCC, USA) to detect any moisture accumulation during the storage period.
4. Use a vacuum heat seal machine (Andrew James, Bowburn, UK) to vacuum and seal the bag.
5. Store γ-irradiated PLGA membranes at a wide range of temperatures from -20°C to +37°C in a moist environment within an incubator containing 5% CO_2 to assess the storage shelf life of the membranes with micro-fabricated ring over 6 months. (The latter condition was chosen to deliberately challenge the packaging and membranes).
6. Post storage check that the vacuum seal remains intact and examine the humidity indicator to confirm that the level of humidity is beneath 30 %. (Any membranes where packaging is not intact or the humidity is greater than 30 % should not be used).
7. Use Scanning Electron Microscopy (SEM) to assess fiber integrity.

3. Isolation of Limbal Explants

Rabbit limbal explants were isolated from rabbit eyes (obtained from, Hook Farm, UK where rabbits are bred for consumption).

1. Disinfect the rabbit eyes using antiseptic solution (Videne 3%, Ecolab, Swindon, UK) and clean the eyes by removing any excess tissue surrounding the cornea.
2. Separate the limbal region from the rest of the cornea and then cut into segments under a dissection microscope. Disinfect the limbal segments in 1.5% videne for 1 min and then cut them into small pieces (100-500 µm) with a scalpel blade.
3. Store the small pieces of tissue (limbal explants) in culture media. (DMEM+Glutamax: Ham´s F12 (1:1), 10% fetal bovine serum, 1U/ml penicillin, 100 mg/ml streptomycin, 2.5 µg/ml amphotericin, 10 ng/ml of EGF and 5 µg/ml of insulin) until use.

3.1. Outgrowth of cells from limbal explants

Rabbit limbal explants were placed on both freshly spun microfabricated membranes and membranes which were vacuum-packed and stored for 6 months at -20^{o}C. Cell outgrowth from tissue explants can be identified by a transient amplifying/stem cell marker, p63 which stains the nucleus to show that the membrane is able to support the growth of limbal epithelial cells.

1. Coat the ring scaffolds with 15 µl of fibrin glue (1:1 mixture of fibrinogen from human plasma (Sigma-Aldrich, UK) at a concentration of 18.75 mg/ml and thrombin from human plasma (Sigma-Aldrich, UK) at a concentration of 2.5 U/ml).
2. Use a cell scraper for distributing the fibrin evenly.
3. Place the tissue explants directly on the PLGA micropockets using a 25 gauge (G) needle and a dissecting microscope.
4. Add cell culture media very gently to avoid detaching the explants.
5. Change media every 3 days and keep in culture for 2 weeks.
6. Fix the samples with 3.7% buffered formaldehyde for 10 minutes followed by 3 washes with PBS.
7. Counterstain by incubation in 1µg/ml of 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich,UK) or propidium iodide (Sigma-Aldrich,UK) for 10 minutes at room temperature.
8. Wash 3 times in PBS and store the samples covered with foil until imaging using a Zeiss LSM 510 META confocal microscope at wavelengths of 543 nm, 800 nm (two-photon) and 488 nm.

4. Setting-up Rabbit Wounded Cornea 3D Models

1. Clean and disinfect the rabbit eyes as described above.
2. Wound the rabbit eyes by immersing them in 0.14% ammonium hydroxide (Sigma Aldrich, UK) for 5 minutes.
3. Rinse the eyes in PBS and scrape away the epithelium with a sclerotome knife.
4. Cut and isolate the cornea-scleral button removing any remaining tissue.
5. Place the corneas epithelial side down onto a sterile cup and fill with 0.5% Agar (Sigma-Aldrich,UK) made in DMEM.

6. Once set, put the corneas, epithelial side up, in small petri dishes and add culture media (recipe described above) up to the limbal area. Do not cover the whole cornea; keep the organ culture at air-liquid interface.

5. Isolation of Limbal Explants and Inclusion of Ring Scaffolds in Rabbit Cornea 3D Models

Positive (corneas without any treatment) and negative controls (wounded corneas) were also maintained in culture for the same periods of time. The negative controls confirmed the lack of formation of a new epithelium in the absence of any added cells.

1. Coat the ring membranes with 15 µl of fibrin glue (1:1 mixture of fibrinogen at a concentration of 18.75 mg/ml and thrombin at a concentration of 2.5 U/ml). Use a cell scraper to distribute the fibrin evenly.
2. Place the tissue explants directly on the PLGA micropockets using a 25G needle and a dissecting microscope.
3. Place the rings with the tissue explants on the deliberately denuded corneas with the explants facing up and at air-liquid interface conditions. These conditions were as previously determined by our group[22].
4. Maintain the organ culture models for 4 weeks in a humidified incubator at 37 ˚C and 5% CO_2.

6. Assessment of corneal regeneration and stem cell maintenance

1. After four weeks, fix the corneas using 3.7% formaldehyde and process them for conventional histology to produce 6 µm paraffin sections (Microtome Leica RM 2145) and then stain with hematoxylin and eosin (H&E).
2. For immunocytochemistry, dewax the sections in xylene and rehydrate in 100% ethanol, 70% ethanol and distilled water.
3. Delineate the sections using a Dako pen to delimit small areas and avoid excessive use of antibody; then treat the delineated areas with 0.05% trypsin (Aldrich) for 20 minutes (37°C).
4. Wash thoroughly with PBS and add 10% goat's serum (blocking) for 1 hour.
5. Incubate the samples with mouse monoclonal antibody cytokeratin 3 (CK3, Merck Millipore) and p63 (Merck Millipore) in 1% goat's serum overnight at 4°C.
6. Wash with PBS and treat with biotinylated secondary anti-mouse antibody (1:1000 in 1% goat serum, Vector Labs) for 1 hour at RT and tertiary antibody FITC-streptavidin (1:100 in 1% goat serum, Vector Labs) for 30 min at RT.
7. Finally, treat the samples with DAPI as described above.

REPRESENTATIVE RESULTS

Electrospun microfabricated rings were manufactured using a combination of microstereolithography and electrospinning (Figures 1 and 2). PEGDA rings of different sizes were fabricated using microstereolithography (Figure 3); this technique allows the fabrication structures in the order of cm and the simultaneous incorporation of microfeatures. In this case we fabricated rings of diameters ranging from 1.2-1.6 cm containing micropockets of 350-500 µm (Figure 4).
In terms of producing, sterilizing and packaging of materials for future clinical use we found that vacuum packing in medical grade bags significantly improved our ability to achieve long term storage of PLGA membranes (Figure 5); the use of a medical grade bag (PET/Foil/LDPE, Riverside Medical Ltd. Derby, UK) with thickness of 0.12 mm allowed us to achieve a longer shelf life. This was investigated by sending membranes to our collaborators in India and membranes were stored for a period of months at -20 °C, at room temperature and at 37 °C in deliberately moist conditions (a moist incubator). Figure 5 shows that using the deliberately provocative conditions of storage at 37 °C under moist conditions, membranes were only stable for approximately 1 month under non-vacuum packed conditions, but achieved 3 months storage under vacuum packed conditions (Figure 5 and Table 1).

Table 1 demonstrates the improvement in storage conditions that can be achieved even under conditions selected to be conducive to water uptake and fiber swelling if one pays attention to the choice of bag used.

The rings supported cell outgrowth from limbal explants in different conditions (i) rings freshly made and (ii) rings stored for 6 months (Figure 6). Cell transfer was achieved after 4 weeks when placing the PLGA membranes on 3D wounded models. Cells grew out from the tissue explants placed on the membranes creating a new epithelium on the previously denuded corneas (Figure 7). Immunocytochemistry demonstrated that the cells coming from the explants were corneal epithelial cells since they were positive for p63 (Figure 6 c & d) after 6 months storage and CK3 (corneal differentiation marker) staining (Figure 7 e).

FIGURE LEGENDS

Figure 1. Schematic of microstereolithography set up for the creation of PEGDA rings.

Figure 2. Schematic of electrospinning process using PEGDA microfabricated rings as a templates.

Figure 3. Figure a shows an example of a static collector (electroplated aluminium sheet with PEGDA rings) for the spinning of microfabricated PLGA membranes. Figures b and e show different electrospun mats being peeled from static collectors. Figure c shows PEGDA templates of different sizes highlighting the versatility of using microstereolithography for the fabrication of the underlying surface. Figure d shows a PLGA microfabricated replica.

Figure 4. SEM image of a PEGDA ring with a horseshoe microfeature (a); high magnification SEM image of a microfabricated pocket (b). Phase contrast image of a PLGA ring with a horseshoe microfeature (a); high magnification phase contrast image of a microfabricated pocket (b).

Figure 5. Effect of temperature and time on storage of vacuum and non-vacuum packed PLGA (50/50) membranes (44 kg/mol) with micro-fabricated rings over 6 months. Membrane integrity was scored as fully intact fibers (+++), some fiber swelling (++), fiber merging (+) or no intact fibers (-). SEM images and three desiccants (silica orange, cobalt (II) chloride and copper (II) sulphate) show no changes in fiber integrity or humidity.

Figure 6. Fluorescence images showing outgrowth of LEC from limbal explants on biodegradable membranes (44 kg/mol) with microfabricated rings on freshly made rings (a,b) and on rings after 6 months storage at -20 °C (c,d). Images a and b correspond to cells stained with DAPI (blue) and propidium iodide (red) respectively. Image b is an orthogonal view from a confocal z-stack of an explant placed on a microfabricated pocket. Images c and d show positive staining for p63 (green).

Figure 7. Figure a shows a rabbit wounded cornea model with a ring scaffold and tissue explants located on the scaffold which was previously coated with fibrin glue. Images b and c are positive and negative controls; the positive control is a fresh rabbit cornea and the negative control a cornea where the epithelium was deliberately removed (the negative control was also cultured for 4 weeks). Figure d is a H&E image of a tissue engineered cornea after 4 weeks in culture; the figure shows the new multi-layered epithelium formed by the cells coming out from the explants placed on the niches. Figure e is an immunocytochemistry image showing cell outgrowth from a limbal explant; nuclei are stained with DAPI (blue) and the cells shows positive staining for cytokeratin 3, a corneal differentiation marker (green).

Table 1. Effect of vacuum and different storage bags on integrity of PLGA (50/50) membranes (44kg/mol) examined over 6 months of storage. Membrane integrity was scored as fully intact fibers (+++), some fiber swelling (++), fiber merging (+) or no intact fibers (-).

DISCUSSION

This study describes (a) a technique for the fabrication of electrospun membranes containing microfeatures within them and (b) how to prepare such membranes for clinical use by vacuum packing, gamma irradiation and then storage prior to use.

In this particular application we have developed PLGA rapidly degrading membranes containing micropockets which mimic the physical features of the limbal stem cell niches. We showed transfer of cells from these ring membranes to a 3D rabbit cornea model by placing limbal explants within the micropockets.

Our group recently reported transfer of cells onto an *in vitro* rabbit cornea model by placing explants on plain PLGA membranes (membranes without ring structures)[22]. Using the present microfabricated scaffolds we have taken cell transfer one step further and we are able to specifically locate tissue explants within the microfeatures providing them with physical protection. Moreover, the ability to place the explants directly within the niches allows the surgeon to use the membranes directly in the surgical theatre avoiding the need of a cleanroom to first expand the limbal stem cells.

In this work we have also explored the storage of the ring membrane over a period of 6 months. Blackwood et al reported that by varying the ratio of PLA to PGA, the degradation of the membrane changes [30]. This study also showed that by increasing the amount of PGA, the degradation rate of electrospun membranes increased *in vivo* [30]. Degradation of the membranes is driven by hydrolysis so by simply keeping the membranes moisture-free the process is halted. In this study we have shown that with vacuum packing the membranes along with some desiccant and irradiating them and storing them at low temperatures for 6 months, there is no change in the fiber integrity and degradation. At present 6 months is as far as we have studied with these membranes but we have reported storage data for 1 year on plain electrospun membranes at -20 °C [22] and we now have unpublished data for their storage at -20 °C for two years without any signs of degradation. Thus for long term storage we would recommend freezing at -20 °C but it is possible to store them at room temperature even in India for at least 6 months (possibly much longer). The inclusion of a humidity indicator gives an easy means of checking that the packaging has kept membranes dry in which case they will be fit for the purpose. In summary, we have created a microstructured membrane using a combination of manufacturing techniques. By placing limbal tissue explants within the microfeatures of the membrane we have shown (i) cell outgrowth from the explants on the niche areas and (ii) cell transfer onto a rabbit wounded cornea and subsequent re-epithelization of the cornea. We have also studied the degradation of the membranes stored at different temperatures and developed a packaging protocol which allows long term storage of membranes and this will be essential in developing membranes for clinic use.

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DISCLOSURES

The authors have nothing to disclose.

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